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**TECHNICAL NOTE**

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# Evaluation of an Automated Liquid Hybridization Method for DNA Quantitation

**ABSTRACT:** The AluQuant™ (Promega Corporation) liquid hybridization DNA quantitation method was evaluated on an automated robotic platform (Biomek® 2000, Beckman Coulter, Fullerton, CA) for use in forensic PCR-STR systems. DNA from bloodstains and buccal swabs was extracted by three different methods: Chelex, Qiagen™ and DNA IQ (Promega). Samples were quantitated using both the Quantiblot and the AluQuant™ systems. Concordance between methods was determined by comparing the average AluQuant™ DNA concentrations for samples having matching (binned) Quantiblot values. Studies testing the "accuracy" (STR analysis), precision, sensitivity, and specifies specificity of the AluQuant™ method were also conducted. The effect of inhibitors (carpet, denim, and suede) was evaluated. The results indicate that the AluQuant™ quantitation system equals the Quantiblot system in "accuracy", sensitivity, precision, and primate-specificity. While extracts from denim and suede affected (inhibited) both systems minimally, the carpet extracts produced a sharp increase in DNA quantitation values in the AluQuant™ but not the Quantiblot system. The speed and user-friendliness of the AluQuant™ system on a robotic platform offer specific advantages to the forensic community.

**KEYWORDS:** forensic science, DNA quantitation, primates, AluQuant™

According to the 2001 U.S. Department of Justice Bureau of Statistics Bulletin, 81% of the nation's publicly operated crime laboratories reported DNA analysis backlogs totaling 16,081 subject cases and 256,329 convicted offender samples (1). Because of this growing demand for DNA analysis, laboratories must begin to adopt techniques that will increase throughput without compromising results. Automation of routine steps in analysis would allow laboratories to devote more time to case management and technical review and, thereby, facilitate throughput.

Polymerase chain reaction assays used in human forensic STR (short tandem repeat) DNA typing require accurate DNA quantitation due to the narrow range of template DNA that is needed for optimal STR multiplex conditions (2). Most forensic biology laboratories employ a slot blot method based on the hybridization of a biotinylated, primate-specific probe (D17Z1) to an alpha-satellite region, followed by either colorimetric or chemiluminescent detection. In this assay, the amount of probe that hybridizes to membrane-bound DNA is directly correlated to the intensity of blue or black colored bands that form on the membrane or film, respectively, after an enzymatic reaction. DNA quantity is determined by visually inspecting the membrane or film and comparing the sample color intensity to that of a set of standards (4,5). This method, more commonly referred to as Quantiblot (PE Applied Biosystems, Foster City, CA), is a fairly accurate and sensitive method, with reported sensitivity as low as 150 pg of human DNA (4). However, the method has several disadvantages: visual inspection introduces the possibility of human error in the interpretation of sample quantity data; the procedure is time consuming and labor intensive; the method does not lend itself easily to automation.

The AluQuant™ Human DNA System (Promega Corporation, Madison, WI) is a liquid hybridization method which uses a probe (proprietary) that is specific to highly repetitive sequences on human chromosomes. Denatured DNA is incubated with the reaction mixture and the probe is allowed to hybridize to the target region. Probe-target hybridization initiates a series of enzymatic reactions: pyrophosphorylation (reversal of DNA polymerization), phosphorylation, and oxidation. READase™ Polymerase recognizes the double-stranded DNA substrate, catalyzes the addition of pyrophosphate across the 3′-terminal bond, and liberates the terminal base as deoxynucleoside triphosphate (dNTP). READase™ Kinase then catalyzes the transfer of the terminal phosphate from the dNTP to adenosine diphosphate (ADP) forming adenosine triphosphate (ATP). The photoprotein, luciferase, catalyzes the ATP mediated oxidation of luciferin to oxyluciferin with the concomitant production of light. The luminescence produced is proportional to the amount of DNA in the sample. Light intensity is read by a luminometer and DNA sample quantity is determined by comparison to a standard curve. This method, with its liquid-driven design, can be run on an automated, robotic platform. The evaluation the AluQuant™ System for use in forensic casework is described herein. We have compared the performance of the AluQuant™ System to that of the colorimetric Quantiblot, the method used by the NYC Office of the Chief Medical Examiner (NYC OCME).

## Materials and Methods

### *DNA Extraction and Quantitation*

Samples from ten bloodstains collected at autopsy by the NYC OCME and ten buccal swabs obtained from employees at the NYC

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OCME were used. Each sample was divided into two portions and the DNA was extracted using the Chelex (6) and Qiagen™ (Qiagen, Valencia, CA) manual extraction methods, respectively. All samples were quantitated using both the slot blot Quantiblot colorimetric technique and the AluQuant™ automated method described below. Quantiblot values reported herein were determined by binning. Thus, DNA quantities (ng) determined using Quantiblot fall within the following categories (bins): 0.15, 0.31, 0.62, 1.25, 2.5, 5.0, and 10. Concordance between the two quantitation methods was determined by comparing the average AluQuant™ DNA concentrations for samples having matching (binned) Quantiblot values.

The AluQuant™ system, due to its liquid driven design, is highly amenable to automation and was evaluated using the Biomek 2000® (Beckman Coulter, Fullerton, CA) robotic platform according to the "80 Sample AluQuant" protocol developed by Promega. The robotic system was controlled by the BioWorks (Beckman Coulter) software developed by Beckman Coulter for the use with the Promega protocol. In each assay 40 samples and six standards were run in duplicate. Samples/standards in the experimental and control (baseline) series were incubated with the probe or nuclease free water, respectively. The standards were prepared by manual dilution of a human genomic DNA standard solution (Perkin Elmer Applied Biosystems, Foster City, CA). With this method 80 pyrophosphorylase/kinase samples and twelve standard reactions could be prepared in two 96 well Robbins plates in approximately 15 min. The plates were sealed with foil and the reactions were incubated at 55°C for 60 min. Samples were cooled to room temperature prior to robotic transfer (Biomek 2000®) to two luminometry plates. The two luminometry plates were manually transferred to the microplate luminometer (Luminoskan Ascent, Thermo Labsystems, Vantaa, Findland) which was controlled through the Ascent Software. Reagents for the luciferase reaction were automatically dispensed and light output was measured in relative light units (RLUs) using the Aluquant.sel program developed by Promega.

Data from the luminometer in RLUs was directly imported into an Excel spreadsheet (Aluguant.xls, Promega) via an interfaced computer. A standard curve was generated and sample concentrations were calculated using a Microsoft Excel® Macro designed by Promega (2). The program calculates the net RLUs in the samples and standards by subtracting the RLUs in the control reactions from those in the experimental. The macro then generates a standard curve by plotting the net RLUs of the standard series vs the DNA concentration. DNA quantity in the samples is determined by comparing the net RLUs in the samples to the standard curve.

DNA extracts from thirty-nine postmortem bloodstains collected by the NYC OCME were also quantitated using the AluQuant™ automated method described above. The samples had been previously extracted using the automated (Biomek 2000) DNA IQ™ (Promega Corporation, Madison, WI) method and quantitated using the Quantiblot method. DNA IQ™ extraction (3) involves a two-step procedure: lysis to release cellular components followed by magnetic capture of the DNA. Both the DNA IQ™ and Qiagen™ methods are compatible with use on an automated or robotic platform. Concordance between the two quantitation methods was determined by comparing the average AluQuant™ DNA concentrations for samples having matching (binned) Quantiblot values.

Certain select samples, quantitated via AluQuant™, were flagged and re-run on AluQuant™ when large differences in the DNA concentration were obtained between Quantiblot and AluQuant™ methods.

The use of these human samples for research purposes was approved by the John Jay College of Criminal Justice and the NYC Department of Health Institutional Review Boards.

*Sensitivity*

Duplicate two-fold serial dilutions were prepared using the standard genomic DNA extract provided in the Quantiblot kit. The series—50, 25, 12.5, 6.25, 3.1, 1.55, 0.75, 0.375, 0.18, 0.09, 0.045, 0.023 and 0.012 ng—were quantitated using the AluQuant™ automated method.

*Species Specificity*

DNA extracts (BIOS Laboratories, New Haven, CT) from chimpanzee, gorilla, macaque, rabbit, rat, mouse, pig, cow, sheep, dog, chicken, frog, fish, and yeast were quantitated using both the Quantiblot and the AluQuant™ automated methods. All extracts were run at a concentration of 2.5 ng/μL with the exception of the chimpanzee which was run at 3.25 ng/μL.

*Reagent Blanks*

Sixty-four extraction reagent blanks (47 Chelex, two Qiagen™ and 15 DNA IQ™) that had been previously quantitated via Quantiblot were quantitated via AluQuant™. This was done in order to establish a lower baseline for calling negative samples in the AluQuant™ system. All the blanks were negative for human DNA when run in Quantiblot.

*Reproducibility*

One Chelex extracted buccal swab sample extract and one Chelex extracted bloodstain sample extract were chosen at random and quantitated twenty times using the AluQuant™ automated method. This procedure was repeated using Qiagen™ extracted buccal and bloodstain sample extracts. Two randomly selected DNA IQ tissue extract samples were quantitated twenty times using the AluQuant™ automated method. Samples had been previously quantitated with both the Quantiblot and AluQuant™ automated methods.

*Inhibition Studies*

Three mm × 3mm cuttings from the following substrates were made: denim blue jeans, leather/suede from a sneaker upper, and a common household indoor/outdoor carpet. All of these material substrates have been shown to be problematic when quantitation is attempted using the Quantiblot system. Inhibitors present within each of these substrates lead to either a complete reaction failure or to a reduced/ heightened sensitivity of the Quantiblot reaction assay.

Each cutting was placed in a separate Eppendorf tube with 1 mL of distilled $H_2O$ and incubated in a 56°C heat block for 60 min. The three tubes were subsequently transferred to a 100°C heat block for 8 min. Two Qiagen™ extracted buccal swab samples were chosen and 1/10 dilutions (sample extract/TE; sample extract/substrate extract) were prepared with each of the three substrates. The inhibitor was added to the sample subsequent to DNA extraction in order to better control the amount of inhibitor present in a sample. The samples were quantitated with both the Quantiblot method and the AluQuant™ automated method.

*DNA Amplification and Genotyping*

Twelve Chelex extracted samples (nine postmortem bloodstain sample extracts and three buccal swab extracts) and 12 Qiagen™ extracted samples (ten postmortem bloodstain sample extracts and

two buccal swab extracts) were amplified. Some samples were selected due to the fact that the quantitation values for the sample were the same or nearly the same across both quantitation systems. These samples were amplified according to this common DNA concentration. Other samples that had been flagged due to a great variation between the AluQuant™ DNA concentration and Quantiblot DNA concentration were also amplified. These samples were amplified twice, according to both the given AluQuant™ DNA concentration and the given Quantiblot DNA concentration. In all cases, the samples were amplified in the Profiler Plus (Perkin Elmer Applied Biosystems) multiplex, for a target of 1 ng of DNA using the GeneAMP® PCR System 9600 (PE Applied Biosystems) according to the manufacturer's instructions. PCR products were electrophoresed using an ABI 377™ DNA Sequencer (Perkin Elmer, Foster City, CA). Results were analyzed using the GeneScan™ and GenoTyper™ (Perkin Elmer) software packages. Peak heights in relative fluorescent units (RFUs) for heterozygotes were calculated by averaging the signals for the two alleles.

## Results and Discussion

Concordant DNA quantitation results between the AluQuant™ and Quantiblot systems were obtained across all sample types and extraction methods. Figures 1–3 plot the average AluQuant™ DNA quantities for samples that were determined to have matching (binned) Quantiblot DNA values for the Chelex ($n = 26$), Qiagen™ ($n = 17$), and DNA IQ™ ($n = 32$) extraction procedures, respectively. AluQuant™ DNA values were more variable at the higher DNA concentrations across all extraction methods. This is to be expected since the range of the binned Quantiblot values is greater at the high than at the lower end. A Quantiblot value of 0.62 ng is equal to or greater than 0.62 but less than 1.25; a value of 1.25 ng is equal to or greater than 1.25 but less than 2.5. Although the number of samples quantitated for each extraction method is small and further testing is required, some trends are apparent. AluQuant™ DNA values for samples in the 0.31 ng Quantiblot bin were most variable for the Qiagen™ extracted samples and least variable for the Chelex extracted samples. AluQuant™ DNA values for samples in the 0.62 ng Quantiblot bin were most variable for the Qiagen™ extracted samples and least variable for those samples extracted by either Chelex or DNA IQ™. Samples in the 1.25 ng Quantiblot bin exhibited more variable AluQuant™ DNA values for samples extracted by the DNA IQ™ method compared to those extracted by either the Chelex or Qiagen™ procedures. Chelex extracted samples showed less variability in AluQuant™ DNA values than those extracted by the DNA IQ™ method for the 2.5 ng binned Quantiblot samples.

The results of the dilution series indicate that the AluQuant™ system is sensitive to DNA levels as low as 0.023 ng (data not shown). This value is comparable to the lower limit (0.15 ng) established for Quantiblot (4).

The results from the animal panel (Fig. 4) demonstrate that the AluQuant™ system is primate-specific. This is an important forensic consideration since any level of contamination, whether it be from animal, bacterial or fungal sources, could lead to an overestimation of the amount of primate DNA in the sample extract if the quantitation system is not primate-specific.

Ten [0.04 ng ($n = 1$), 0.03 ng ($n = 2$), 0.02 ng ($n = 2$), and 0.01 ng ($n = 5$)] out of the 64 Quantiblot reagent blanks did not give a 0.00 ng reading in AluQuant™ (data not shown). Thus, a proposed baseline suggested by these results is 0.02 ng. All negative samples with quantitation values greater than 0.02 ng would be re-run to confirm the absence/presence of DNA.

The precision of the AluQuant™ system was evaluated by using two randomly selected sample extracts from each extraction system (Chelex, Qiagen™ and DNA IQ™) and quantitating each sample

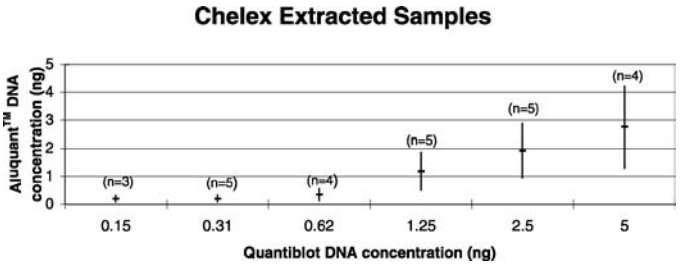


FIG. 1—*Average AluQuant™ DNA quantities obtained for Chelex extracted samples having matching (binned) Quantiblot values. Bars indicate one standard deviation.*

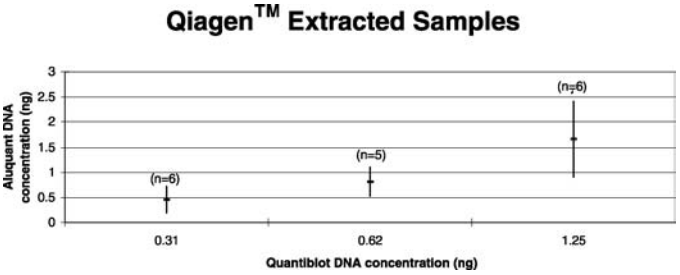


FIG. 2—*Average AluQuant™ DNA quantities obtained for Qiagen™ extracted samples having matching (binned) Quantiblot values. Bars indicate one standard deviation.*

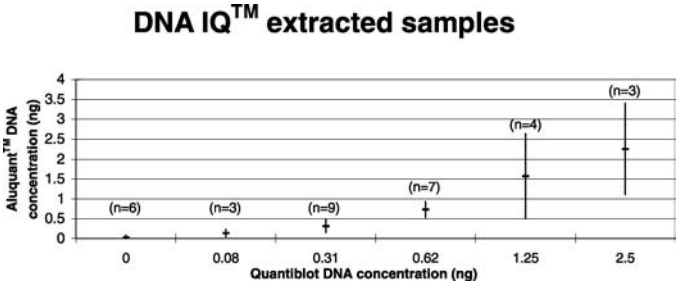


FIG. 3—*Average AluQuant™ DNA quantities obtained for DNA IQ™ extracted samples having matching (binned) Quantiblot values. Bars indicate one standard deviation.*

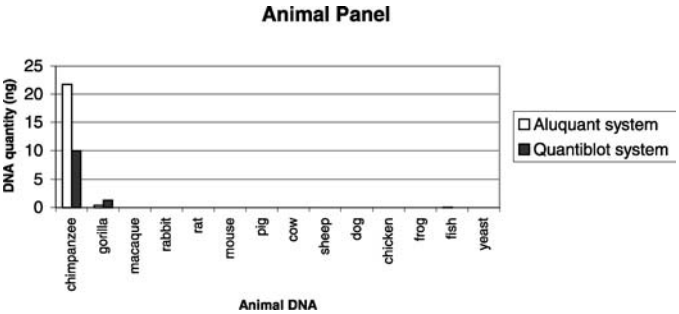


FIG. 4—*Quantitation of various animal (and yeast) DNA extracts using the AluQuant™ and Quantiblot systems. All extracts contained 12.5 ng with the exception of the chimpanzee which contained 17.5 ng.*

TABLE 1—*Precision of the AluQuant system based on twenty replicate runs for each sample.*

| Sample Type | Extraction Method | Quantiblot Value (ng) | Average AluQuant Value (ng) ± Standard Deviation | 95% Confidence Interval |
|---|---|---|---|---|
| Oral Swab | Chelex | 1.25 | 1.39 ± 0.30 | 0.59 |
| Bloodstain | Chelex | 1.25 | 1.13 ± 0.19 | 0.37 |
| Oral Swab | Qiagen | 0.62 | 0.67 ± 0.09 | 0.18 |
| Bloodstain | Qiagen | 1.25 | 1.53 ± 0.27 | 0.53 |
| Bloodstain | DNA IQ | 1.25 | 1.66 ± 0.53 | 1.04 |
| Bloodstain | DNA IQ | ND* | 1.27 ± 0.23 | 0.45 |

* ND, not determined.

extract twenty times (see Table 1). As expected, variability was greater for those samples in the higher Quantiblot bin. Overall the results indicate that the reproducibility of AluQuant™ system is adequate for forensic purposes. However, studies using a larger range of DNA quantity and sample type are needed to more fully evaluate the reliability of the AluQuant™ system.

Results from the inhibition studies indicate that the AluQuant™ system does not offer any advantage over the Quantiblot system when quantitating DNA from stains mixed with blue jean or suede-leather substrate extracts (data not shown). Addition of the blue jean extract resulted in a sharp decline in quantitation values for both samples quantitated by AluQuant™ and for one sample analyzed by Quantiblot. Addition of the sneaker extract reduced the quantitation value for one sample analyzed by AluQuant™; Quantiblot results were not affected. In contrast, the addition of the carpet extract to DNA samples adversely affected AluQuant™ but not Quantiblot system. Sharp increases in DNA concentration were observed (data not shown) when the carpet extract was added. Therefore, it is hypothesized that a component(s) in the carpet extracts caused the sharp increase in RLU values and the concomitant increase in concentration values. Since the number of samples and types of substrates used in the inhibition study was small, further comparisons need be conducted on larger sample sizes and with more varied substrates. It would be interesting to test the effect of the carpet extracts on the luciferase reaction.

Eight samples that had been flagged due to a variation between the AluQuant™ DNA concentration and Quantiblot DNA concentration were amplified and typed in the Profiler Plus system. Interestingly, all of these samples (blood and buccal) had been extracted using either the Chelex or Qiagen methods. In most cases, the differences seen in DNA concentration between the AluQuant™ concentration and the Quantiblot concentration was no greater than three-fold, and typically closer to a two-fold difference. These samples were amplified twice, according to both the given AluQuant™ DNA concentration and the given Quantiblot DNA concentration. Peak height analysis at three loci (vWA, D8S1179, D5S818) is shown in Table 2.

The majority of the samples exhibited similar behavior. For example, in a case where a sample (sample 4) was recorded as having a 0.125 ng/μL concentration in the AluQuant™ system and a 0.25 ng/μL concentration in the Quantiblot system, the sample was both amplified at both 8 μL and 4 μL volumes, respectively. Peak heights were slightly higher for the sample amplified according to the AluQuant™ concentration (1000 RFU at the vWA locus as compared with 500 RFU), which could be explained by more initial input of DNA. As expected, samples amplified using equal amounts of target DNA (sample 1) had virtually equal RFU values. In contrast, the RFU values obtained for equal input DNA concentrations in Sample 2 were disparate: two-fold higher for the reaction based on the AluQuant™ vs the Quantiblot value. This discrepancy is most likely due to differences in DNA concentration. The subjective nature of the Quantiblot, particularly in visualizing faint bands corresponding to the smaller DNA concentrations, could have resulted in lower DNA input. Thus, differences in peak height for samples with "identical" Quantiblot values (Samples 1 and 2) reflect the range of DNA concentrations contained within a particular bin and the subjective nature of the Quantiblot technique (Sample 2).

For a given DNA concentration (0.25 ng/μL), results for the AluQuant™ samples (Samples 3 and 6) were less variable than those for the Quantiblot samples (Samples 4, 7 and 8). Overall, there were no negative stochastic effects, no major peak imbalances in the heterozygotes, and none of the samples appeared either under or over amplified. Since the differences seen in Quantiblot and AluQuant™ concentrations were generally not greater than a two-fold difference, these results indicate that the input amplification volumes did not differ to a large degree. This leads to the conclusion that the AluQuant™ system exhibits a degree of "accuracy" approaching that of the Quantiblot system.

TABLE 2—*Correlation between the amount of template DNA and peak height.*

| Sample | AluQuant™ Concentration (ng/μL) | Quantiblot Concentration (ng/μL) | Volume (μL) of Extract Required For 1 ng of Target DNA (μL) | Peak Height (RFU*) At vWA Locus | Peak Height (RFU) At D8S1179 Locus | Peak Height (RFU) At D5S818 Locus |
|---|---|---|---|---|---|---|
| 1 | 0.31 | | 3.3 | 350 | 350 | 800 |
| | | 0.0625 | 16.0 | 400 | 250 | 800 |
| 2 | 0.125 | | 8.0 | 1500 | 1500 | 700 |
| | | 0.0625 | 16.0 | 600 | 600 | 300 |
| 3 | 0.25 | | 4.0 | 3000 | 2000 | 2000 |
| | | 0.125 | 8.0 | 4500 | 3000 | 2500 |
| 4 | 0.125 | | 8.0 | 1000 | 2500 | 2000 |
| | | 0.25 | 4.0 | 500 | 1000 | 600 |
| 5 | 0.0625 | | 16.0 | 950 | 900 | 1100 |
| | | 0.125 | 8.0 | 800 | 900 | 1000 |
| 6 | 0.25 | | 4.0 | 1500 | 1000 | 950 |
| | | 0.125 | 8.0 | 3000 | 2000 | 1500 |
| 7 | 0.125 | | 8.0 | 4500 | 4000 | 3500 |
| | | 0.25 | 4.0 | 3000 | 4000 | 4000 |
| 8 | 0.625 | | 1.6 | 1000 | 1000 | 1500 |
| | | 0.25 | 4.0 | 2500 | 3000 | 3000 |

* Relative fluorescence unit.

The results of these studies indicate that the AluQuant™ quantitation system equals the Quantiblot system in sensitivity, "accuracy", precision, and primate-specificity. In addition, the speed and user-friendliness of the AluQuant™ system on a robotic platform such as the Biomek 2000 offer specific advantages to the forensic community. Automation of routine steps in DNA analysis would facilitate throughput of the steadily increasing number of samples submitted to laboratories and help to alleviate the DNA analysis backlog.

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